

APPENDIX B

Attorney Docket No. 021149.000001

Appl. No. : 10/516,381
 Applicants : Guenther Eissner, et al.
 Filed : June 10, 2005
 Art Unit : 1635
 Conf. No. : 4749
 Examiner : Amy Hudson Bowman
 Docket No. : 021149.000001
 Customer No. : 24239
 Title : Method for Protection of Endothelial and Epithelial Cells During Chemotherapy

Declaration Under 37 C.F.R. §1.132

Honorable Commissioner of
 Patents and Trademarks
 Washington, D.C. 20231

Sir:

GUENTHER EISSNER declares that:

1. He is a co-inventor of and is familiar with the present U.S. patent application Serial No. 10/516,381, filed June 10, 2005 in the name of Guenther Eissner and Ernst Holler and entitled "Method for Protection of Endothelial and Epithelial Cells During Chemotherapy" and is familiar with the Official Action dated May 23, 2008 issued therein and with the prior art references cited in the Official Action, including the Barcoglu et al. (U.S. Patent No. 3,624,912), Sayer et al. (*J. Cancer Res. Clin. Oncol.*, March 2002, 128, pgs. 148-152), Balrey et al. (*American Journal of Hematology*, April 2002, 69, pgs. 281-284), and De Luca et al. (*Int. J. Cancer*, 1997, 73, pgs 277-282) references.

2. He received a Bachelor of Science degree in Human Biology from the Philipps-University of Marburg-Germany in 1988 and a Ph.D. from the Institute for Immunology of the Ludwig-Maximilians-University of Munich Germany in 1992. From 1992 to 1997, he served his post-doctoral fellow at the Institute for Clinical Molecular Biology of the GSF-Research Center for Environment and Health, in Munich Germany. From 1997 to 1998, he was employed with Ludwig-Maximilians-University of Munich, Germany, from 1998 to 2004 he was employed at the University of Regensburg, Germany, from 2004 to 2007 he was employed with Gentium, S.p.A., and from 2008 to the present time he has been employed at the Grosshadern Medical Center-University of Munich, Germany as a Professor for Interdisciplinary Stem Cell Research in the Department of Cardiac Surgery. His primary area of expertise comprises the field of Immunology, with particular emphasis on transplantation and stem cell biology. He is a co-inventor on two Patent Cooperation Treaty applications and has authored numerous publications and grants in the field of immunology, including stem cell biology and transplantation science.

3. Under his direction and control, the following experiment was performed:

Materials and Methods: The human dermal microvascular endothelial cell line CDC/EU-HMEC-1 (HMEC-1) was kindly provided by the Centers for Disease Control and Prevention

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(Atlanta, GA) and has been established as previously described.¹ HMECs were cultured in MCDB131 medium supplemented with 10% fetal calf serum (FCS), 1 µg/mL hydrocortisone (Sigma, Delsenhofen, Germany), 10 ng/mL epidermal growth factor (Collaborative Biobchemical Products, Bedford, MA), and antibiotics. All cell culture reagents were purchased from Gibco BRL (Karlsruhe, Germany) unless stated otherwise. 5-Fluorouracil (5-FU) was obtained from Sigma (Delsenhofen, Germany). Defibrotide was obtained from Gentium SpA (Villa Guardia (CO), Italy).

Apoptosis Assay: An established method for detecting apoptosis in human endothelial cells was performed as previously described using flow cytometry (FACScan and CellQuest software (Becton Dickinson/Pharmingen, Heidelberg, Germany)).² Endothelial and tumor cells were left untreated or were incubated in the presence of 5-FU in descending concentrations (range: 10 µg/mL to 0.1 µg/mL) in the presence or absence of defibrotide or oligotide³ for 48 hours. Cells were then washed in phosphate-buffered saline (PBS) + 10% FCS and were stained with the necrosis-detecting dye propidium iodide (PI; 0.2 µg/mL; Sigma, Delsenhofen, Germany). Apoptotic cells were identified by PI-negative staining and by a characteristic side scatter (SSC) image distinct from that of non-apoptotic cells. At least 3 experiments per cell type were performed.

Results: Results obtained from the experiment are presented below in Figure 1 and demonstrate emblematic for the protective effects of an oligonucleotide of the present invention on a patient's epithelial and/or endothelial cells from immunosuppressant-induced apoptosis and/or activation. Specifically, the data clearly shows that the addition of 5-FU induces apoptosis in HMECs. However, administering either defibrotide or oligotide⁴ counteracted the 5-FU-induced apoptosis, thereby achieving protection of the endothelial cells from the apoptosis induced by the immunosuppressant.

¹ Ades, G.W., Candel, F.J., Sverlick, R.A. et al. "HMEC-1: Establishment of an immortalized Human Microvascular Endothelial Cell Line." *J. Invest. Dermatol.* 1992; 99:683-690.

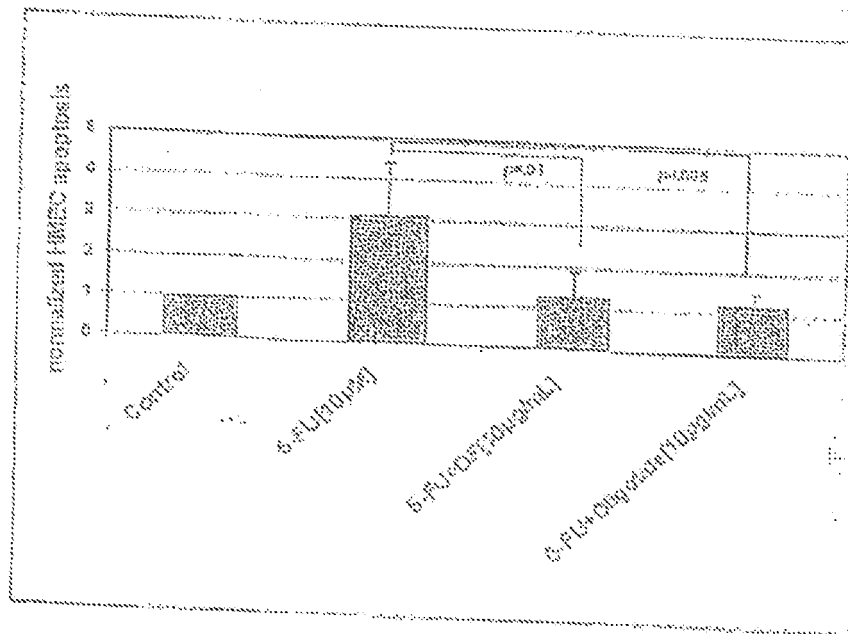
² Coner, T.G., Lemmon, S.V., Glynn, J.M., Green, D.R. "Microfilament-Disrupting Agents Prevent the Formation of Apoptotic Bodies in Tumor Cells Undergoing Apoptosis." *Cancer Res.* 1992; 52:1997-1999.

³ The "oligo" as used in these experiments had the following physico-chemical and elemental characteristics: MW: 4600-10,000 Da; hyperchromicity parameter: < 10; A+T/C+G: 1.100-1.455; A+G/C+T: 0.800-1.160; specificity: mutation: +30±4%.

⁴ *Id.* footnote 3.

Attorney Docket No. 021149.0000001

Figure 1



These results demonstrate that both defibrinide or oligonucleotide are capable of preventing 5-FU-mediated apoptosis in HMEC cells, and therefore satisfies the enablement requirement as it pertains to the terms "protective oligonucleotide" and "immunosuppressant."

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Guenther Klaser

3 Nov 2008
Date

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